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Ion channel activity of HIV-1 Vpu is dispensable for counteraction of CD317☆

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ABSTRACT

While the C-terminal domain of HIV-1 Vpu is critical for CD4 degradation, the transmembrane domain (TM) mediates ion channel activity, enhances virus release and is essential for counteracting CD317/Bst-2/Tetherin. Here we analyzed whether the ion channel activity of Vpu is required to antagonize CD317-mediated restriction of virion release. We examined TM-mutants of three conserved residues: the S23A mutation, which was previously shown to abrogate ion channel function, did not affect Vpu mediated augmentation of virus release. In contrast, the A14N and A18N mutation did not affect ion channel activity of Vpu, but substantially reduced its ability to support virus release and to down-regulate CD317 from the cell surface. Altogether, our data suggest that not the ion channel activity of Vpu, but its ability to remove CD317 from the cell surface is required to augment HIV-1 release.

Introduction

The human immunodeficiency virus type 1 (HIV-1) accessory protein Vpu is an 81 amino acid oligomeric type 1 integral membrane phosphoprotein (Cohen et al., 1988; Maldarelli et al., 1993; Strebel et al., 1988), which is encoded exclusively in HIV-1 and related simian immunodeficiency viruses (SIV), but not in HIV-2. Vpu has been shown to induce degradation of the CD4 receptor by the ER-associated protein degradation (ERAD) pathway (Schubert et al., 1992; Schubert and Strebel, 1994; Willey et al., 1992a,b) and enhances virus particle release from the plasma membrane (Gottlinger et al., 1993; Klimkait et al., 1990). Both functions can be genetically dissected (Schubert and Strebel, 1994). The cytoplasmic domain of Vpu contains a pair of serine residues (at positions 52 and 56), which are constitutively phosphorylated by the casein kinase 2 (CK-2) (Schubert et al., 1994). The phosphorylation of these two serine residues in the cytoplasmic domain is critical to induce CD4 degradation (Friborg et al., 1995; Schubert et al., 1992, 1994; Schubert and Strebel, 1994). In contrast, mutations within the transmembrane (TM) domain of Vpu retain the ability to degrade CD4, but abrogate the ability to enhance virion release (Schubert and Strebel, 1994). In addition, the TM domain of Vpu was found to form a non-selective, voltage gated ion channel (Ewart et al., 1996; Schubert et al., 1996b). Randomization of the TM domain prevents Vpu's ion channel formation and impairs its ability to regulate virus release (Schubert et al., 1996a). This suggested a causal relation between the ion channel activity of Vpu and its augmentation of virus release. However, the importance of Vpu's ion channel activity for virus release has neither been experimentally demonstrated nor mechanistically supported.

Recently, it was shown that Vpu enhances HIV-1 virion release by counteracting a cellular restriction factor termed CD317 (also known as tetherin, BST-2 or HM1.24) (Neil et al., 2008; Van Damme et al., 2008). CD317 is an interferon-induced restriction factor, which tethers virions to the cell surface, thereby impairing the release of HIV-1, HIV-2, SIV and other enveloped viruses (Kaletsky et al., 2009; Mansouri et al., 2009; Neil et al., 2007; Sakuma et al., 2009; Zhang et al., 2009). CD317 is a type 2 TM protein and is targeted to cholesterol-rich lipid rafts in the plasma membrane, the preferential site for HIV-budding (Kupzig et al., 2003). CD317 also localizes in the endosomal system, including the trans-Golgi network (Rollason et al., 2007). CD317 has a unique topology, consisting of a 21 amino acid long cytoplasmic tail, a TM domain with a membrane-spanning α-helix, a 110 amino acid ectodomain and a C-terminal glycosyl phosphatidylinositol (GPI) anchor. The latter mediates the localization into lipid rafts (Hinz et al., 2010; Ishikawa et al., 1995; Perez-Caballero et al., 2009). Recently, it was shown that the ectodomains of CD317 form parallel dimers and that these homodimers tether virions to cellular membranes (Yang et al., 2010). Furthermore, CD317 is constitutively recycled through endosomal compartments (Varthakavi et al., 2006).

Vpu and CD317 interact *via* their TM domains and this interaction is required for efficient surface down-regulation of CD317 (Banning et

☆ Function of Vpu ion channel.

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al., 2010; Dube et al., 2010; Iwabu et al., 2009; Rong et al., 2009). Surface down-regulation and intracellular degradation of CD317 induced by Vpu occurs *via* lysosomes or proteasomes (Douglas et al., 2009; Goffinet et al., 2009; Iwabu et al., 2009; Mangeat et al., 2009; Mitchell et al., 2009). Vpu recruits the ß-transducin repeat-containing protein (ß-TrCP), a subunit of the Skp1-Cullin-F-box (SCF) E3 ubiquitin ligase complex, which binds to phosphorylated Ser-52 and Ser-56 in the cytoplasmic domain of Vpu (Margottin et al., 1998; Mitchell et al., 2009), and thereby the SCF E3 ubiquitin ligase complex induces ubiquitination of CD317 on serine and threonine residues in the cytoplasmic domain of CD317. This step is required for efficient down-regulation of CD317 from the cell surface (Tokarev et al., 2011). However, Vpu does not increase the rate of internalization of CD317, suggesting that Vpu acts at a post-endocytotic step to reduce cell surface levels of CD317 (Douglas et al., 2009; Dube et al., 2010; Mitchell et al., 2009). It has been shown that CK-2 inhibitors block the degradation of CD317 by Vpu (Schindler et al., 2010), however, mutation of the ß-TrCP binding motif only partially affects the virion release (Douglas et al., 2009; Mitchell et al., 2009; Schubert et al., 1994, 1995) and does not induce a specific re-localization of CD317 from the cell surface into a perinuclear compartment (Dube et al., 2010). Recently, it was demonstrated that mutation of several alanine residues, in particular the A14L-, A18H-, and to a lesser extent also A18L-mutation impairs Vpu mediated counteraction of CD317 (Skasko et al.; 2011; Vigan and Neil, 2010).

In order to unravel the importance of the ion channel activity for the ability of Vpu to enhance virus release, we mutated the conserved Ala-14 and Ala-18 in the TM domain of Vpu and analyzed the resulting mutants for their ability to form ion channels and to counteract CD317. Mutation of Ala-14 and Ala-18 to asparagine impairs the efficiency of surface down-regulation of CD317. However, both mutants still exhibit ion channel activity in 293T cells, indicating that channel activity of Vpu is not sufficient to support virus release. Furthermore, to assess its relevance in CD317 counteraction, we mutated Ser-23, which is essential for Vpu's ion channel activity, to alanine (S23A) (Mehnert et al., 2008). Intriguingly, the S23A mutant still efficiently interacted with CD317, and thus supported virus release in the presence of CD317. Taken together, our data suggest that the ion channel activity of Vpu is not critical for counteraction of CD317.

Results and discussion

The VpuTM mutants A14N and A18N do not support virus release in the presence of CD317.

Vpu has two main structural domains, the membrane-spanning hydrophobic N-terminal TM domain and the C-terminal cytoplasmic domain. While the TM domain is important for anchoring the protein to the cellular membrane and to enhance virus release, the cytoplasmic domain is crucial for the induction of CD4 degradation (Bour et al., 1995; Schubert et al., 1994, 1995; Chen et al., 1993; Friborg et al., 1995; Schubert and Strebel, 1994), and also to lesser extent for degradation of CD317 (Douglas et al., 2009; Mitchell et al., 2009; Schindler et al., 2010). Nevertheless, both functions can be genetically separated (Schubert and Strebel, 1994). A Vpu mutant containing a randomized TM sequence (RD) is incapable of supporting virion release, as binding to CD317 is abrogated, but retained *wt* activity for CD4 degradation (Banning et al., 2010; Schubert and Strebel, 1994). The RD mutant contains a randomized TM sequence with a conserved amino acid content and α-helical structure. The RD mutant is integrated normally into membranes, it is able to form homo-oligomers, it exhibits expression levels, protein stability and sub-cellular localization similar to those of the *wt* Vpu (Schubert et al., 1996a). However, it was shown that the RD mutant lost ion channel activity and was unable to antagonize CD317 (Paul et al., 1998; Schubert et al., 1996b; Tiganos et al., 1998). Hence, after scrambling the amino acid sequence, a dissection of the two mechanisms, ion channel formation and CD317 antagonism, is not possible.

Thus, in order to analyze whether ion channel activity of Vpu correlates with viral particle release, several TM mutants were generated. It was previously shown that Ser-23 of Vpu is essential for ion channel activity (Mehnert et al., 2008). Therefore, we generated a specific Vpu mutant, containing a point mutation in which Ser-23 is replaced by alanine (Fig. 1A).

Recently, it has been demonstrated that several highly conserved residues in the TM domain of Vpu, particularly Ala-18, Ala-14 and Trp-22 are involved in counteraction of CD317 (Skasko et al., 2011; Vigan and Neil, 2010). This stretch of conserved residues, which are located on one face in the α-helix of the TM domain, regulates binding of Vpu to CD317 and is highly conserved among Vpu proteins derived from HIV-1 group M and N isolates (Skasko et al., 2011; Vigan and Neil, 2010). However, this particular TM motive is absent in Vpu proteins derived from HIV-1 group O isolates, which are defective in CD317 antagonism, but retain the ability to down-regulate CD4 from the cell surface (Sauter et al., 2009; Vigan and Neil, 2010). To analyze the function of the TM domain of Vpu in CD317 down-regulation, we mutated Ala-18 and Ala-14 to Asn in Vpu from the HIV-1 NL4-3 sequence (A18N; A14N). The A18N, A14N and S23A mutants were introduced into the pΔR vector, an HIV-1_{NL4-3} based expression construct that produces noninfectious virus like particles (VLPs), but expresses authentic viral transcripts in the correct ratios (Gottwein and Krausslich, 2005). HeLa cells, which constitutively express endogenous CD317, were transfected with pΔR *wt*, ΔVpu, A18N, A14N or S23A plasmids. 24 h post transfection VLPs were collected by centrifugation and the amount of released virions was determined by Western blot analysis (Fig. 1B). As expected, virus release was severely reduced in the absence of Vpu (Fig. 1B lane 2). The A18N and A14N mutations also substantially reduced the ability of Vpu to support virus release (Fig. 1B, C), again confirming that both alanine residues are important for counteracting CD317 (Skasko et al., 2011; Vigan and Neil, 2010). In contrast, mutation of Ser-23 has no effect on viral particle release, indicating that the loss of channel activity does not disturb CD317 counteraction (Skasko et al., 2011; Vigan and Neil, 2010). To confirm these results, 293T cells were co-transfected with pΔR or pΔRΔVpu and Vpu was complemented in *trans* with a plasmid expressing either AU1-tagged Vpu *wt* or indicated mutants thereof, together with Flag-tagged CD317. Overexpression of the A14N and A18N mutants could not restore virus release, again confirming that Ala-14 and Ala-18 are required to antagonize CD317 (Fig. 1D, E). Overexpression of the S23A mutant substantially enhances virus release in presence of CD317, similar to *wt* Vpu (Fig. 1D, E).

Vpu TM-mutants localize at the plasma membrane

Vpu was shown to localize in the ER, Golgi, endosomal structures and at the plasma membrane (Klimkait et al., 1990; Pacyniak et al., 2005; Varthakavi et al., 2006). In order to rule out the possibility that mutations of Ala-18 and Ser-23 affect localization of Vpu at the plasma membrane, cell surface biotinylation analysis was performed. This is particularly important, as it was shown previously that mutation of Ala-18 to His (A18H) resulted in abnormal retention of Vpu in the ER (Hout et al., 2006; Skasko et al., 2011). 293T cells were transfected with either AU1-tagged Vpu, A18N or S23A and Flag-tagged CD317, as a control. 24 h post transfection cell surface proteins were biotinylated with Sulfo-NHS-LC-biotin for 15 min at 4 °C. Cells were subsequently lyzed and biotinylated proteins were immunoprecipitated with immobilized Streptavidin, separated by SDS-PAGE and analyzed by Western blot. As shown in Fig. 2, CD317, Vpu and both TM mutants were efficiently immunoprecipitated with Streptavidin. The ribosomal P0 antigen, which localizes in the cytoplasm, is not detectable after immunoprecipitation. Hence, in contrast to the

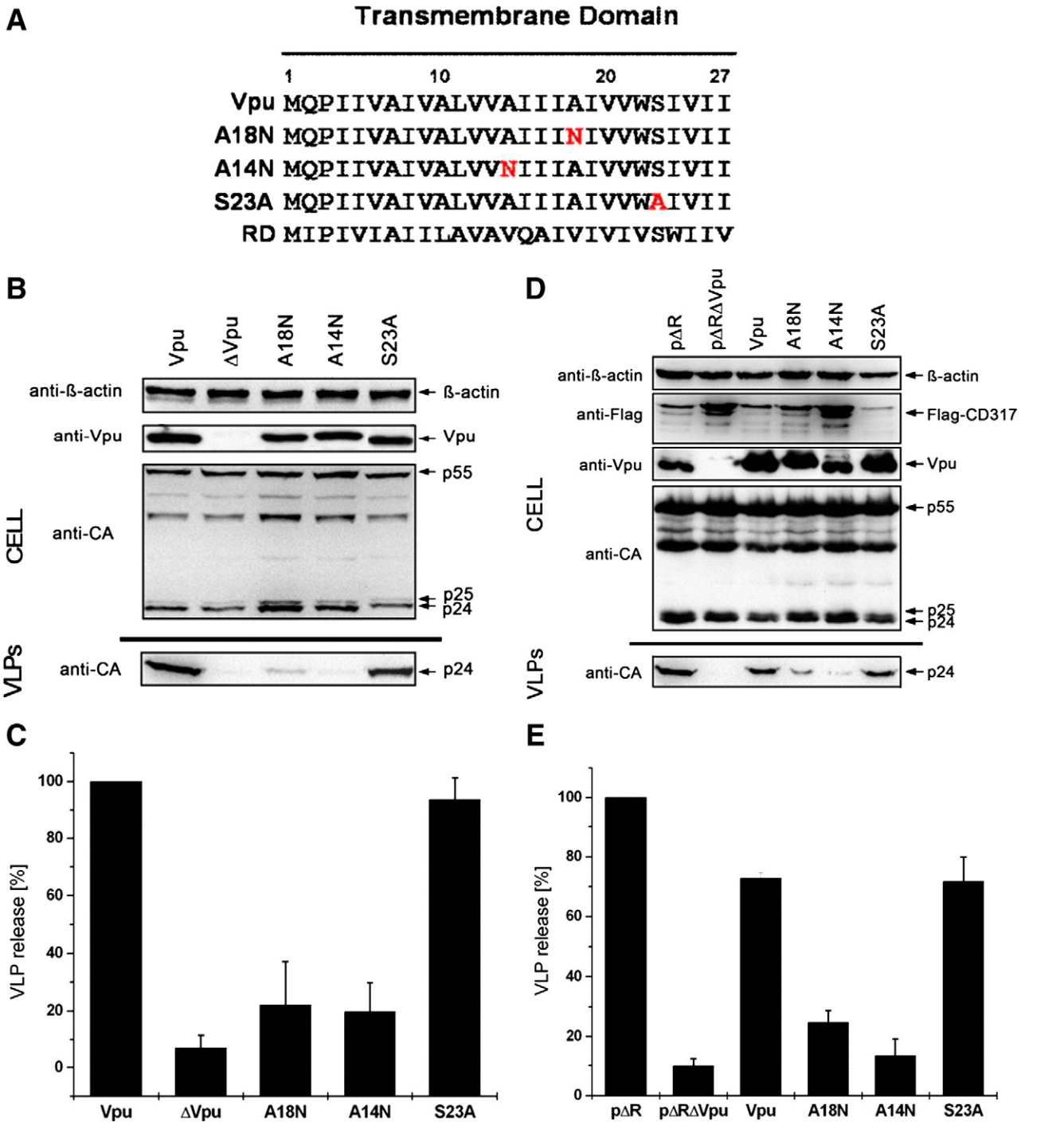

Fig. 1. The ability of Vpu TM mutants to antagonize CD317. (A) Schematic representation of the amino acid sequence of the transmembrane domain of Vpu *wt*, A18N, A14N, S23A and RD. (B) A18N and A14N mutations abrogate augmentation of virus release. HeLa cells were transfected with either pΔR *wt*, ΔVpu, A18N, A14N and S23A plasmids. 24 h post transfection VLPs were pelleted and the amount of released virions was determined by Western blot. In addition, cells were lyzed and analyzed by Western blot, using anti-Vpu and anti-CA antibodies. (C) Quantification of Gag release of 3 independent experiments. (D) Overexpression of the A18N and A14N mutants together with CD317 substantially reduced virion release. 293T cells were co-transfected with either pΔR, pΔRΔVpu or pΔRΔVpu complemented in trans with a plasmid expressing either Vpu, A18N, A14N and S23A and Flag-tagged CD317. 24 h post transfection VLPs were pelleted and the amount of released virions was determined by Western blot. In addition, cells were lyzed and analyzed by Western blot, using anti-Vpu, anti-CA- and anti-Flag antibodies. (E) Quantification of Gag release of 3 independent experiments.

A18H mutation (Skasko et al., 2011), the A18N exchange does not affect trafficking of Vpu to the plasma membrane.

The A18N mutant exhibits a normal sub-cellular distribution as wt Vpu

To investigate whether the A18N mutant exhibits a normal sub-cellular distribution as *wt* Vpu, we analyzed the sub-cellular localization of *wt* Vpu, the RD, the A18H and the A18N mutants by confocal microscopy. HeLa cells were transfected with Vpu-YFP fusion proteins (Banning et al., 2010), containing the mutations A18H, A18N or RD. 24 h post transfection the cells were fixed, permeabilized and stained for Calnexin, a Ca^{2+}-binding protein that is primarily located in the ER. Confirming recent results (Skasko et al., 2011), the A18H mutant is retained in the ER, leading to efficient co-localization with

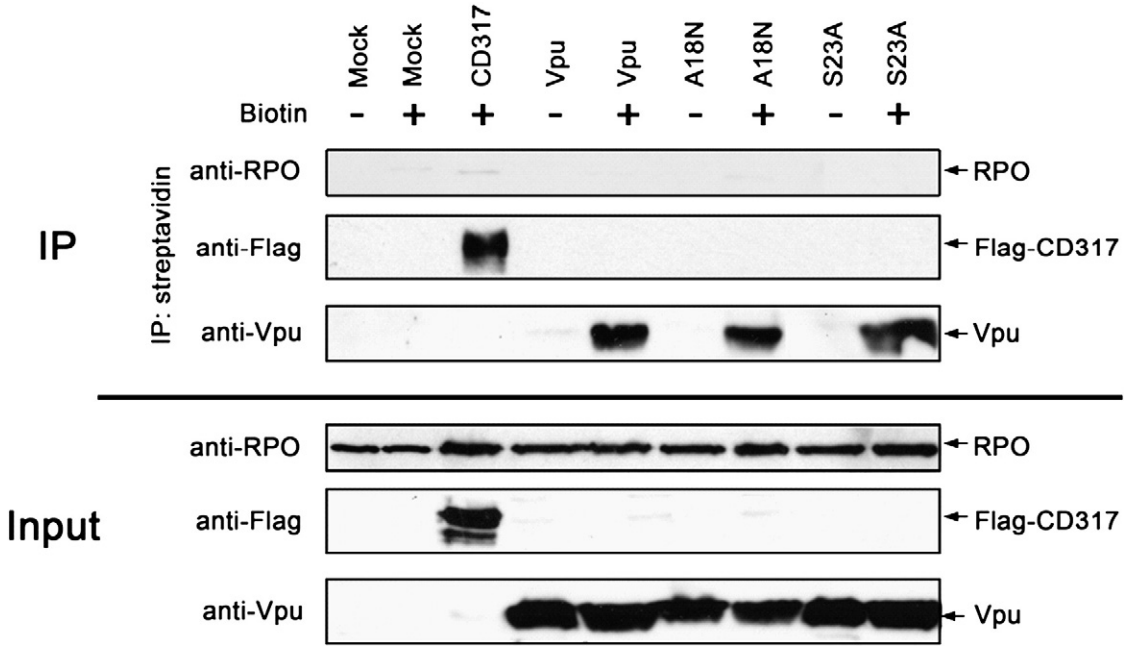

Fig. 2. Surface biotinylation of Vpu and TM mutants. 293T cells were transfected with either Flag-tagged CD317 or AU1-tagged Vpu *wt*, A18N or S23A. 24 h post transfection, cell surface-expressed proteins were biotinylated. Upon cell lysis, biotinylated proteins were captured with immobilized streptavidin, separated by SDS-PAGE and analyzed by Western blot. Vpu and CD317 were detected, using polyclonal anti-Vpu and anti-Flag antibodies.

Calnexin (Fig. 3A). This could be confirmed by surface biotinylation analysis, which revealed substantially reduced levels of the A18H mutant at the plasma membrane compared to *wt* Vpu (data not shown). In contrast, the A18N and RD mutants co-localized less extensively with Calnexin, showing similar to Vpu *wt* a more endosomal sub-cellular distribution. To confirm an ER retention of the A18H mutant, cells were treated with the ER transport inhibitor Brefeldin A (BFA). BFA is a fungal metabolite that blocks protein traffic from the ER to the Golgi system and blocks the Vpu-dependent enhancement of virion release, but not the Vpu-induced decay of CD4 (Schubert and Strebel, 1994). In the presence of BFA, the A18N, A18H and RD mutants as well as Vpu *wt* are retained in the ER and co-localized efficiently with Calnexin (Fig. 3B). Overall, these data show that in contrast to the A18H mutant, the A18N mutation does not affect sub-cellular distribution of Vpu.

Monitoring ion channel activity of Vpu and Vpu TM mutants analyzed by patch-clamp measurements

In silico studies of Vpu indicate that the TM domain of Vpu forms pentameric structures (Lopez et al., 2002; Moore et al., 1998), and oligomerization of the TM domain of Vpu has been shown to result in the formation of ion channels (Ewart et al., 1996; Schubert et al., 1996a). To test the A18N mutant of Vpu for its ability to form ion channels, the protein was expressed in 293T cells and the membrane currents of transfected cells were then recorded with the patch-clamp technique in the whole cell configuration. The Vpu *wt* protein, which was extensively studied in several systems, served as a positive control (Ewart et al., 1996; Schubert et al., 1996b). The following three negative controls were employed: first, untransfected 293T cells; second, the RD mutant, which lacked channel activity in *Xenopus laevis* oocytes and planar lipid bilayers (Schubert et al., 1996b); and third, the S23A mutant, which did not show channel activity after reconstitution in lipid bilayers (Mehnert et al., 2008). The data shown in Fig. 4B demonstrate that the level of all Vpu constructs in the plasma membrane of the cells was indeed comparable.

Untransfected 293T cells exhibited the typical electrical characteristics of these cells with a small outward rectifier and a very small inward current. A typical measurement of an untransfected cell with the corresponding current/voltage (IV) relation is shown in Fig. 4A. Characteristic for all untransfected cells was a high ratio of steady current at +80 mV and the current at −140 mV. The mean ratio I_{+80mV}/I_{-140mV} obtained from 5 untransfected cells is summarized in Fig. 4C. The same high ratio I_{+80mV}/I_{-140mV} reflecting a minute inward current and some outward rectifier was observed in cells expressing both, the S23A mutant or the RD mutant (Fig. 4A, C). This implies that these proteins did, as expected, not generate any conductance in 293T cells. Cells expressing Vpu *wt* exhibit an overall larger conductance. In particular the inward conductance in these cells was much larger than in the negative controls (Fig. 4A). As a result of a Vpu generated conductance, the I/V relation of these cells became quasi linear. This is reflected in a robust low I_{+80mV}/I_{-140mV} ratio (Fig. 4C). Cells expressing the A18N or A14N mutants exhibit the same electrical features as those expressing Vpu *wt*, i.e., they have a higher inward current and a small I_{+80mV}/I_{-140mV} ratio (Fig. 4C). The exemplary current responses with the corresponding current/voltage curves of 293T cells expressing Vpu *wt* or the A18N or A14N mutants are shown in Fig. 4A. The results of these experiments show that the A18N and A14N mutants are functional channels and generate membrane conductance in the same way as the *wt* protein. Therefore, we conclude that ion channel activity of Vpu is dispensable for counteraction of CD317, as the A18N mutant does not support virus release in presence of CD317 (Fig. 1), however, it still generates ion channel activity.

Binding of Vpu TM mutants to CD317

It was demonstrated that Vpu binds to CD317 via its TM domain, and that this binding is essential to antagonize CD317 (Banning et al., 2010; Rong et al., 2009). Furthermore, it was previously shown that mutation of Ala-14 to leucine abrogates the ability of Vpu to interact with CD317 (Vigan and Neil, 2010). To investigate whether the A14N, A18N or the S23A mutations influence the interaction of Vpu and CD317, co-immunoprecipitation experiments were performed. 293T cells were co-transfected with plasmids expressing AU1-tagged Vpu and mutants thereof, together with Flag-tagged CD317. 24 h post

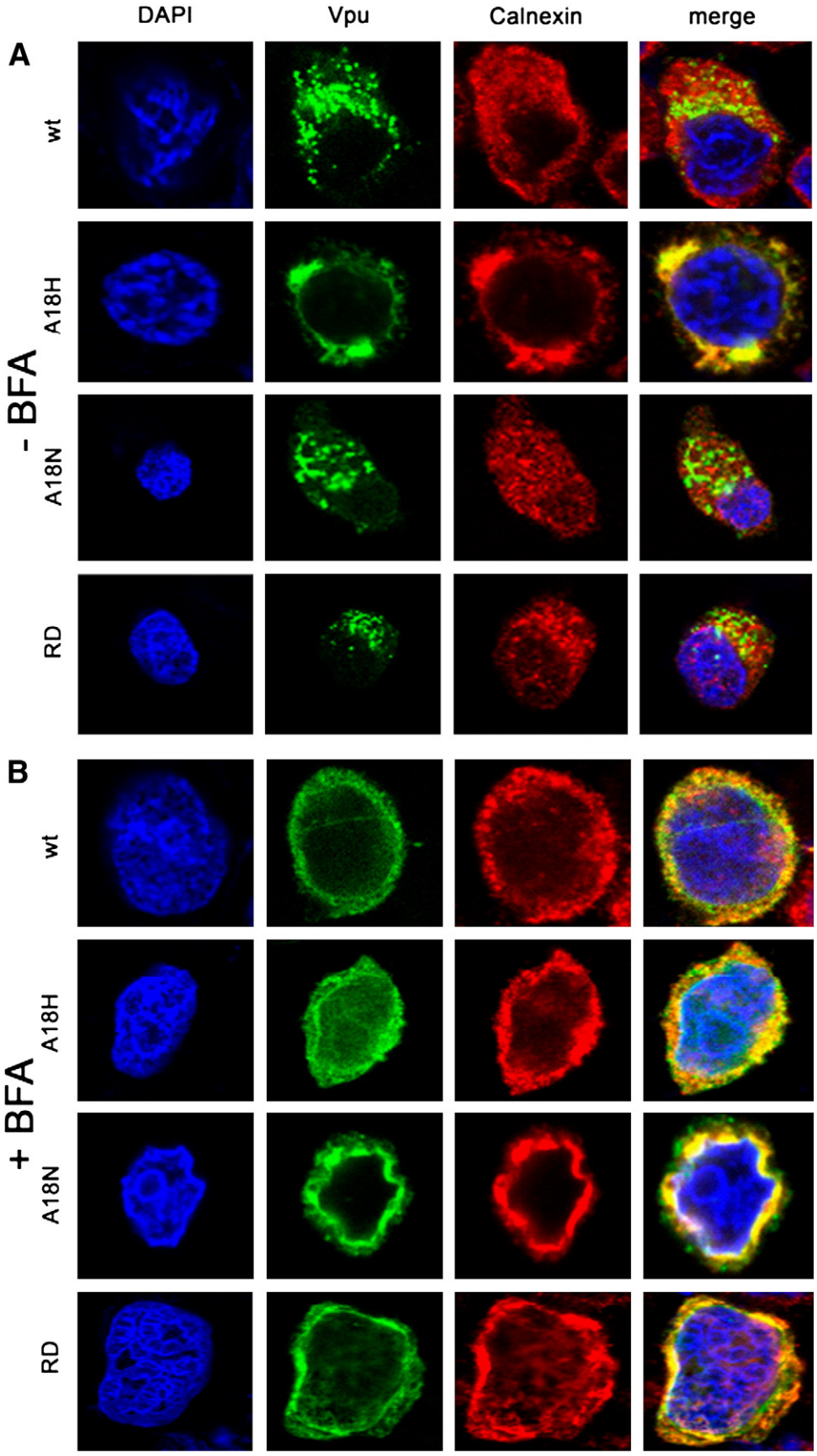

Fig. 3. Sub-cellular distribution of Vpu TM mutants in presence or absence of Brefeldin A. HeLa cells were transfected with either Vpu *wt*, A18H-, A18N- or RD-YFP fusion proteins (green) either in absence (A) or presence (B) of BFA. 5 h after transfection, BFA (10 μg/ml) was added to the cells and 24 h post transfection the cells were fixed, permeabilized and stained for Calnexin (red).

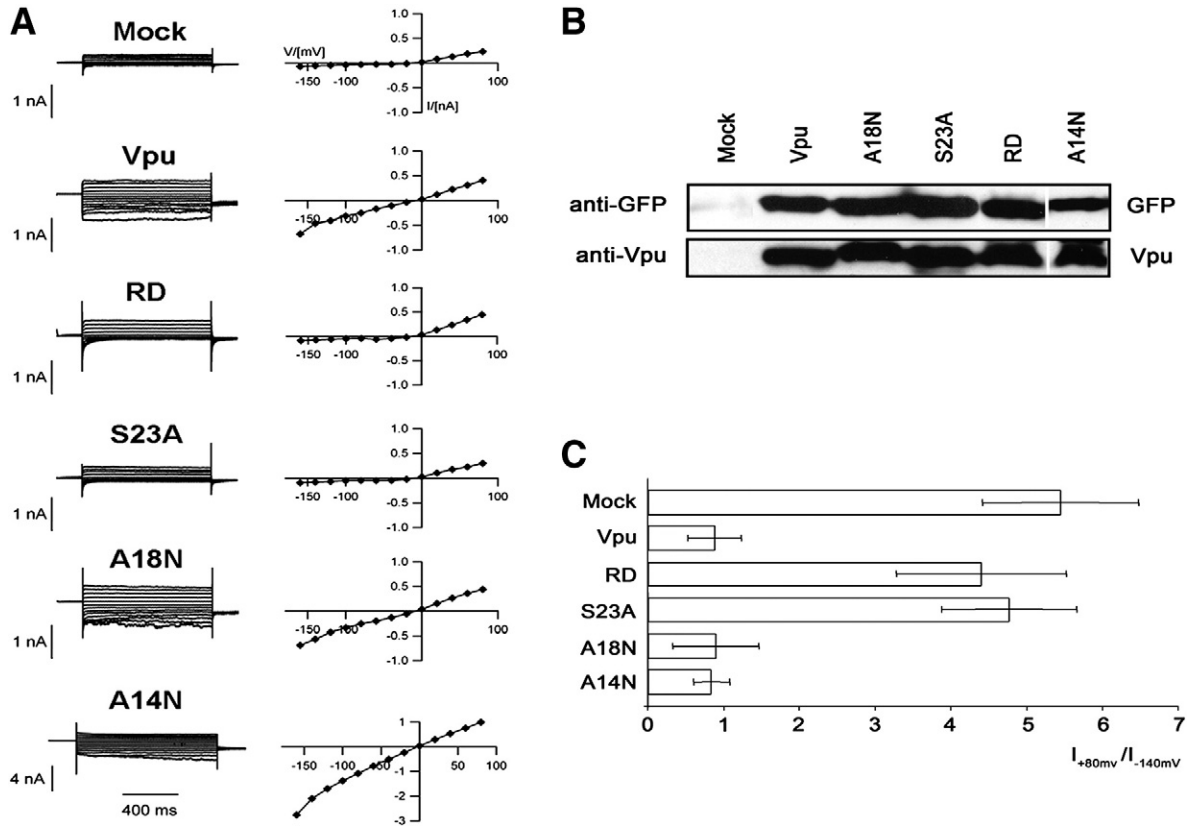

Fig. 4. The ability of Vpu and TM mutants to form ion channels. (A) Exemplary current responses of 293T cells transfected with either AU1-tagged Vpu- *wt*, RD-, S23A-, A18N or A14N-IRES-GFP, respectively (left side) and corresponding steady-state I/V relationships (right side). Currents were measured in whole cell configuration in a 50 mM KCl containing bath solution and elicited by standard voltage protocol from holding voltage (0 mV) to test voltages between +80 and −160 mV. (B) Cells were lyzed and protein expression was analyzed by Western blot, using anti-Vpu and anti-GFP antibodies. (C) Ratio of steady state currents at +80 mV and −140 mV (I_{+80mV}/I_{-140mV}) of 293T cells transfected with either Vpu *wt*, RD, S23A, A18N or A14N, respectively. Values are the means of $n = 5$ cells ± SEM.

transfection cells were lyzed and Vpu was immunoprecipitated, using a monoclonal anti-AU1 affinity matrix. Immunoprecipitated material was analyzed for the presence of CD317 by Western blot, using Flag-specific antibody. CD317- and Vpu expression was detected with anti-Flag and anti-Vpu-serum, respectively. As shown in Fig. 5A and in agreement with previous data (Banning et al., 2010, Rong et al., 2009), CD317 was efficiently co-immunoprecipitated with *wt* Vpu, but not with the RD mutant (Fig. 5A, lanes 4, 7). As expected, the S23A mutant, which is able to antagonize CD317 (Fig. 1), efficiently binds CD317 (Fig. 5A, lane 6). In agreement with recent data (Vigan and Neil, 2010), the A14N mutation abrogates the interaction to CD317 (Fig. 5A, lane 12). Notably, the A18N mutant still binds to CD317, however, it appears to be attenuated when compared to Vpu *wt*.

To confirm whether mutations of Ala-18 affect the interaction between Vpu and CD317, we employed a recently developed FACS-based Försters resonance energy transfer (FRET) assay (Banning et al., 2010) to determine the binding of Vpu and CD317 in living cells (Fig. 5B). To this end, a CFP-CD317 plasmid was co-transfected with a Vpu-YFP plasmid or TM mutants in 293T cells. In control experiments, the S52A phosphorylation mutant, containing a functional TM domain, did not reduce the frequency of FRET positive cells (Banning et al., 2010). In contrast, the RD mutant expressed-together with CD317- showed a strongly diminished FRET signal, and mutation of Ala-18 to His in Vpu impaired binding of Vpu to CD317. This is consistent with recent results, which demonstrated a reduced interaction between Vpu A18H and CD317 (Skasko et al., 2011). Interestingly, the A18N mutant showed also a strongly reduced FRET signal similar to the RD mutant, indicating that binding to CD317 is attenuated (Fig. 5B). To confirm these results, Vpu-YFP fusion proteins and Flag-tagged CD317 were co-immunoprecipitated with anti-Vpu serum, separated by SDS-PAGE and analyzed by Western blot, using anti-Flag and anti-GFP antibodies (Fig. 5C). Vpu and the A18H mutant were efficiently immunoprecipitated with CD317, but not the RD mutant (Fig. 5C, lanes 5, 7, 8). Compared to the A18H mutant, binding of the A18N mutant to CD317 is even more attenuated. Although still detectable in the IP (Fig. 5C, lane 6), the interaction is apparently too weak to be detected by FRET (Fig. 5B). Taken together, these data indicate that Ala-18 in Vpu somehow participates in the binding to CD317. However, while mutations of Ala-14 and Trp-22 almost completely eliminate binding of Vpu to CD317 (Vigan and Neil, 2010, and data not shown), mutation of Ala-18 merely attenuates binding. Yet, it is still not clear whether this defect in binding alone is the underlying reason for the inefficient counteraction of CD317 by the A18N mutant.

Vpu A18N induces degradation of CD4, but is impaired in down-regulation of CD317 from the cell surface

Although the precise mechanism by which Vpu counteracts CD317 is still unclear, it was shown that augmentation of virus release by Vpu correlates with its ability to down-regulate CD317 from the cell surface (Mitchell et al., 2009; Rong et al., 2009; Skasko et al., 2011; Tokarev et al., 2011). In order to investigate whether the TM mutations affect the ability of Vpu to down-regulate cell surface levels of CD317, HeLa cells were transfected with plasmids expressing Vpu *wt* or TM mutants along with GFP translated via an internal ribosomal entry site (Vpu-IRES-GFP) to monitor transfected cells. 24 h post transfection cells were harvested, stained for cell surface CD317, using a CD317-specific monoclonal antibody and analyzed by flow cytometry. Expression of Vpu proteins was controlled by Western blot

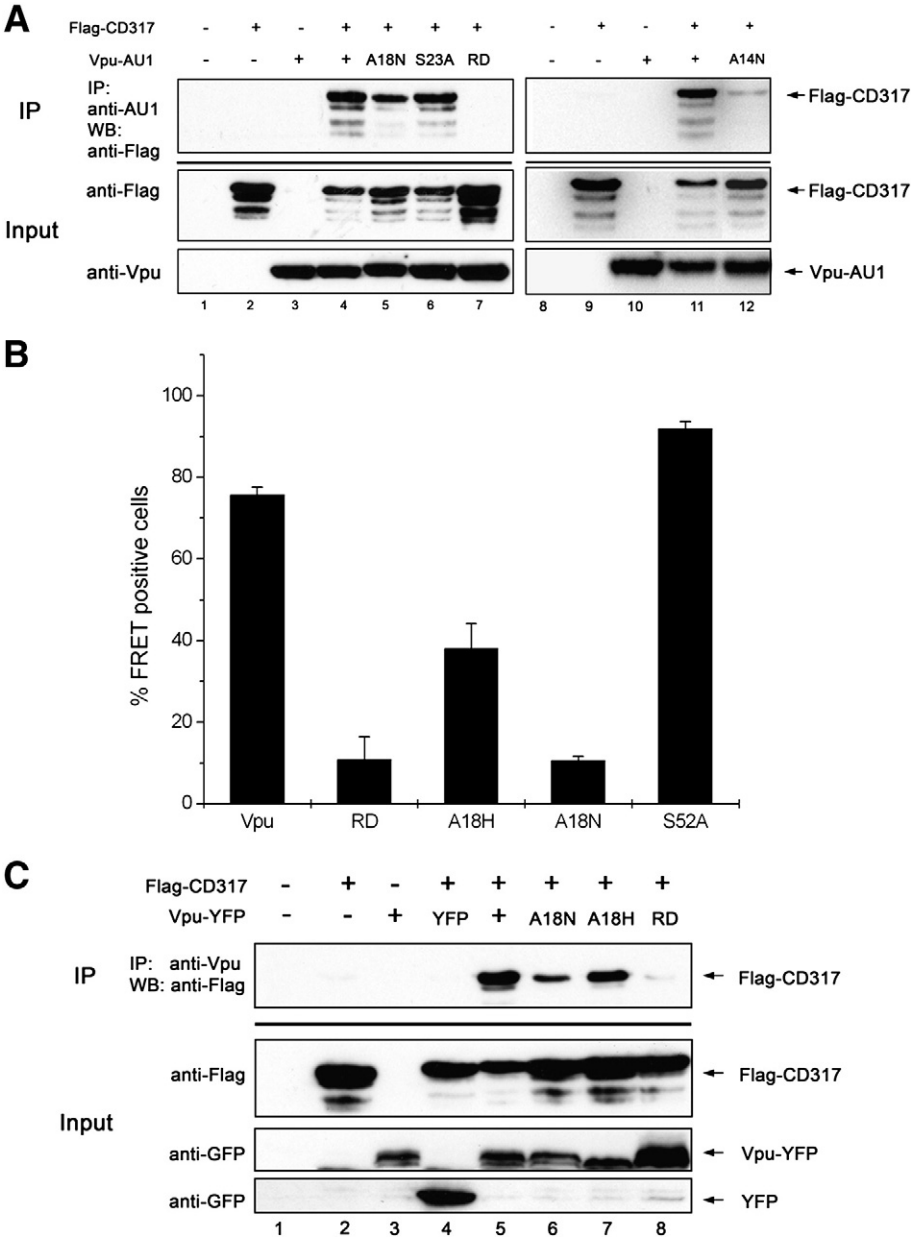

Fig. 5. Interaction of Vpu and TM mutants with CD317. (A) 293T cells were co-transfected with AU1-tagged Vpu- *wt*, A18N-, RD-, S23A- or A14N-IRES-GFP and Flag-tagged CD317. 24 h post transfection cells were lyzed and Vpu immune complexes were isolated from cell detergent extracts by immunoprecipitation with monoclonal AU1 affinity matrix and analyzed for the presence of CD317 by Western blot with anti-Flag antibody. CD317- and Vpu expression were detected with either anti-Flag or anti-Vpu antibody. (B) FACS-based FRET analysis of 293T cells transfected with YFP-Vpu- and CFP-CD317 expression plasmids. Mean and SD are derived of two independent experiments with duplicate transfections. (C) 293T cells were transfected with the indicated Vpu-YFP fusions and a FLAG-tagged CD317. Vpu immune complexes were isolated from cell detergent extracts by immunoprecipitation, using anti-Vpu rabbit serum and analyzed for the presence of CD317 by Western blot with anti-FLAG. YFP-fusion proteins were detected with anti-GFP antibody.

(Fig. 6A, top). The surface expression of CD317 in Vpu expressing cells was calculated as mean fluorescence intensity (MFI) of CD317 expression in GFP positive cells relative to GFP negative cells. Fig. 6A shows that Vpu, as well as the S23A mutant, efficiently down-regulate the cell surface expression of CD317 approximately by 4-fold (Fig. 6A). As expected, the RD mutant completely failed to induce down-regulation of CD317 from the cell surface. In contrast, the A18N mutant and the A14N mutant (data not shown) were substantially attenuated in down-regulation of CD317 (Fig. 6A). GFP positive cells expressing the A18N mutant displayed similar amounts of CD317 at the cell surface when compared to GFP negative cells. Nonetheless, some down-regulation was detected at high GFP expression levels, indicating that the mutant retains some activity at high expression levels (Fig. 6A).

Vpu induces CD4 degradation by the ERAD pathway *via* recruiting ß-TrCP, a subunit of the SCF E3 ubiquitin ligase complex (Schubert et al., 1992, 1994; Willey et al., 1992a,b). In order to confirm that the introduced TM mutations in Vpu selectively interfere with CD317 down-regulation and do not render the protein completely inactive, we examined the ability of the A18N and S23A mutants to induce down-regulation of CD4. CD4 positive HeLa cells were transfected with Vpu-IRES-GFP plasmids encoding for Vpu *wt* or VpuTM mutants. 24 h post transfection the cells were harvested and stained for surface CD4, using a monoclonal CD4-specific antibody and analyzed by flow

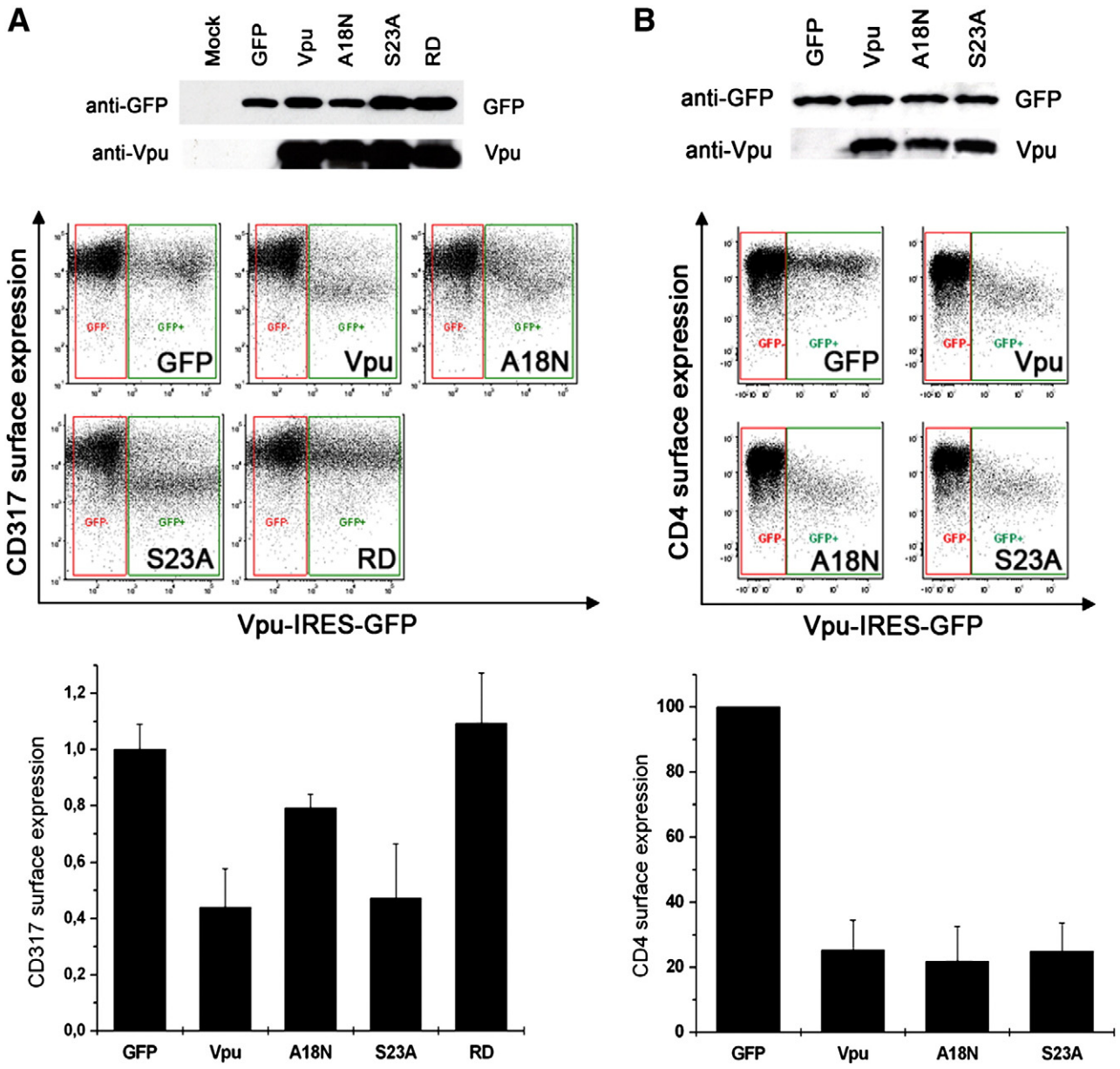

Fig. 6. The A18 mutant is specifically defective in CD317 antagonism. (A) HeLa cells were transfected with either Vpu-IRES-GFP or TM mutants. 24 h post transfection cells were harvested and stained for surface CD317, using a monoclonal CD317-specific antibody and analyzed by flow cytometry. In addition, cells were lyzed and analyzed by Western blot, using anti-GFP and anti-Vpu antibodies. Summary of relative CD317 surface expression from 3 independent experiments is given below. (B) CD4 positive HeLa cells were transfected with either GFP, AU1-tagged Vpu-IRES-GFP or VpuTM mutants. 24 h post transfection cells were harvested and stained for surface CD4, using a monoclonal CD4-specific antibody and analyzed by flow cytometry. In addition, cells were lyzed and analyzed by Western blot, using anti-GFP or anti-Vpu antibodies. Summary of relative CD4 surface expression from 3 independent experiments is given below.

cytometry. Comparable expression levels of all Vpu proteins were confirmed by Western blot analysis (Fig. 6B). The data confirm that all TM mutants efficiently induced surface down-regulation of CD4, indicating that the cytoplasmic domains of the mutants are functional.

Taken together, our results show that the ion channel activity of Vpu is dispensable for counteraction of CD317. The ability of Vpu to enhance virus release directly correlates with its ability to down-regulate cell surface CD317, but not with its ion channel function.

Until now, no biological role for the ability of Vpu to form ion channels has been established so far. Nevertheless, the ion channel might still be important for other functions of Vpu. Very recently, Vpu was shown to downmodulate NTB-A (SLAMF6) on infected T cells, thereby protecting the infected cells from lysis by natural killer (NK) cells (Shah et al., 2010). Yet, the precise mechanism how Vpu down-regulates NTB-A has not been unraveled so far. Furthermore, it was shown that Vpu inhibits cell surface expression of CD1d in dendritic cells (DCs), thereby retaining the protein in early endosomal compartments (Moll et al., 2010). In both novel functions of Vpu, the role of the ion channel activity of Vpu has not been investigated so far. Thus, although well established, the ion channel activity of Vpu might be important for some still unknown functions of Vpu.

Materials and methods

Site-directed mutagenesis and plasmid constructions

All plasmids containing HIV-1 sequences are derivatives of the infectious molecular clone pNL4-3 (Adachi et al., 1986). The pΔR plasmid contains a primer binding site deletion and a reverse transcriptase (RT) active-site mutation and produces noninfectious

VLPs (Gottwein and Krausslich, 2005). Mutations were introduced by site-directed mutagenesis (Quick change Kit, Stratagene), using oligonucleotides, containing indicated changes. For cloning of pΔRΔVpu a 205-bp EcoRI–NheI fragment from the pNL4-3vpuDEL-1 carrying a 41-bp out-of-frame deletion in vpu (Klimkait et al., 1990), was introduced into the pΔR expression vector. Mutations were also introduced into the previously described pCG-IRES-GFP vector, expressing AU1-tagged Vpu together with GFP from a bicistronic mRNA (Sauter et al., 2009; Schindler et al., 2003) and the pEYFP-Vpu plasmid, encoding a Vpu-YFP fusion protein (Banning et al., 2010). The pCMV-Flag-CD317 expression vector was described elsewhere (Banning et al., 2010).

Cells culture and transfection

HeLa cells and HEK293T were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 mg/ml streptomycin.

For transfection, 9×10^5 cells/well were seeded in 6-well plates 1 day prior to transfection. The next day transfection was performed using Lipofectamine 2000™ (Invitrogen), according to the manufacturer's protocol.

Virus release assay

HeLa cells were transiently transfected with either pΔR or pΔRΔVpu complemented in *trans* with a plasmid expressing either Vpu, A18N, A14N or S23A, using Lipofectamine 2000™. Cells and virions were harvested 24 h post transfection. Cells were lyzed in RIPA-buffer (1% NP-40, 0.5% Na-DOC, 0.1% SDS, 0.15 M NaCl; 50 mM Tris–HCl pH 7.4; 5 mM EDTA) and analyzed by Western blot. Cells and cell debris from virus containing supernatant were removed by centrifugation at 1000×*g* for 5 min and 8000×*g* for 10 min. Virions were purified by centrifugation through 20% sucrose at 20,000×*g* for 90 min, re-suspended in 1 ml of PBS, pelleted again for 20,000×*g* for 90 min to remove serum albumins and finally analyzed by Western blotting.

Co-immunoprecipitation assay

For co-immunoprecipitation, 9×10^5 293T cells were transfected with either 150 ng pCMV-FLAG-CD317 or 1 μg AU1-tagged Vpu and TM mutants or Vpu-YFP fusions proteins and TM mutants expression plasmids, as indicated. Cells were lyzed in digitonin lysis buffer (140 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, protease inhibitor mixture (Roche), 1% (w/v) digitonin (Calbiochem)), in order to solubilize transmembrane proteins. Cleared lysates were incubated with monoclonal AU1 affinity matrix (Covance) or anti-Vpu-serum for 90 min on ice. Immune complexes were washed four times in wash buffer (lysis buffer with 0.1% digitonin), separated in 12% SDS-PAA gel and analyzed by Western blot with M2-anti-FLAG-HRP antibody (Sigma). Vpu expression was detected, using a polyclonal anti-Vpu antiserum (rabbit), directed against the hydrophilic C-terminal domain of Vpu.

FACS-based FRET-assay

Flow cyometric measurement of FRET and the according gating strategy were performed as already described (Banning et al., 2010). Briefly, 9×10^5 293T-cells were seeded in a 6-well plate and transfected, using the calcium phosphate method. Cells were harvested and assayed 1 day post transfection for FACS-FRET analysis in a FACSAria (BD Bioscience). ECFP and FRET were measured by exciting cells with the 405 nm laser and ECFP-emission was collected with a standard 450/40 filter, while FRET was measured with a 529/24 filter. EYFP was excited with the 488 nm laser and emission was collected with a 529/24 filter. At least two thousand CFP/YFP positive cells were analyzed for FRET.

Flow cytometric analysis of Vpu mediated CD317 and CD4 down-regulation

HeLa cells were transfected with pCG-Vpu-AU1-IRES-GFP or TM mutants. 24 h post transfection the cells were harvested and stained for surface CD317, using an anti-CD317 monoclonal antibody (Abnova) and chicken anti-mouse Alexa647 conjugated secondary antibody (Molecular Probes, Invitrogen). CD317 surface expression on GFP positive cells was then analyzed in a FACS LSRII, using *FACSDiva* software (BD Biosciences). Data analysis was performed, using FCS Express V3 software (De Novo).

CD4 positive HeLa cells were transfected with either pCG-Vpu-AU1-IRES-GFP or Vpu TM mutants. 24 h post transfection the cells were harvested and stained for surface CD4, using an anti-human CD4 APC conjugated antibody (Invitrogen). CD4 surface expression on GFP positive cells was then analyzed in a FACS LSRII, using *FACS*Diva software (BD Biosciences). Data analysis was performed, using FCS Express V3 software (De Novo).

Cell surface protein biotinylation

Cell surface proteins were biotinylated with EZ-Link Sulfo-NHS-LC-Biotin according to the manufacturer's protocol (Thermo scientific). Briefly, cells were washed three times with ice-cold phosphate-buffered saline (PBS), and primary amines of the membrane proteins exposed to the exterior of the cells were biotinylated with Sulfo-NHS-LC-biotin for 15 min at 4 °C. The cells were washed and lyzed with RIPA buffer. Biotinylated proteins were precipitated at 4 °C with immobilized Streptavidin (Thermo Scientific Pierce). Precipitated material was washed four times in RIPA buffer, separated in 12% SDS-PAA gel and analyzed by Western blot, using polyclonal anti-Vpu antiserum (rabbit), M2-anti-FLAG-HRP antibody (Sigma) for detection of CD317 and human anti-Ribosomal P antibody (Immunosion, INC) to detect ribosomal P antigen.

Immunoflourescence microscopy

HeLa cells were transfected with either Vpu *wt*, A18H-, A18N- or RD-YFP fusion proteins. 5 h after transfection BFA (10 μg/ml) was added and 24 h post transfection the cells were fixed in 3% formaldehyde, permeabilized, using 0.1% Triton X-100 in PBS and stained for Calnexin, using anti-Calnexin antibody (Acris). Images were obtained using a Leica TCS SP5 confocal microscope with the 63× objective and analyzed by Adobe Photoshop software.

Electrophysiological measurements

293T cells were transiently transfected with AU1-tagged Vpu or TM mutants, using liposomal transfection reagent TurboFect™ (Fermentas). 24 h post transfection cells were washed with PBS, dispersed with Accutase® (SIGMA-Aldrich) and seeded into new culture dishes with lower density. For the electrophysiological measurements, the culture medium was removed and replaced by bath solution. The bath solution contained 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 50 mM KCl. The pH was adjusted to 7.4 with KOH. The osmolarity was adjusted to 330 mOsmol with mannitol. The pipette solution contained 130 mM D-potassium-gluconic acid, 10 mM NaCl, 5 mM HEPES, 0.1 mM guanosine triphosphate (Na salt), 0.1 μM $CaCl_2$, 2 mM, $MgCl_2$, 5 mM phosphocreatine and 2 mM adenosine triphosphate (ATP, Na salt); the pH was adjusted to 7.4 with KOH, the osmolarity was adjusted to 330 mOsmol with mannitol.

Single cell patch-clamp measurements were performed at room temperature in whole-cell configuration, using standard methods (Hamill and Marty, 1981) with an EPC-9 patch-clamp amplifier (HEKA, Lamprecht, Germany. Cells were clamped from a holding voltage (0 mV) to test voltages between +80 and −160 mV and back to post potential (−80 mV). The data were acquired and analyzed with the Pulse software (HEKA, Lamprecht, Germany).

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